

ARBOR ASSAYS™
Interactive Assay Solutions™



DetectX®

High Sensitivity Direct Cyclic AMP Chemiluminescent ELISA Kit

1 Plate Kit Catalog Number K019-C1

5 Plate Kit Catalog Number K019-C5

Species Independent

Sample Types Validated:

**Cell Lysates, Saliva, Urine, EDTA and
Heparin Plasma, Tissue Culture Media**

Please read this insert completely prior to using the product.
For research use only. Not for use in diagnostic procedures.

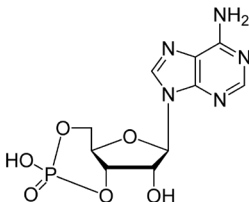
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BACKGROUND

Adenosine-3',5'-cyclic monophosphate, or cyclic AMP (cAMP), $C_{10}H_{12}N_5O_6P$, is one of the most important second messengers and a key intracellular regulator. Discovered by Sutherland and Rall in 1957¹, it functions as a mediator of activity for a number of hormones, including epinephrine, glucagon, and ACTH²⁻⁴. Adenylate cyclase is activated by the hormones glucagon and adrenaline and by G protein. Liver adenylate cyclase responds more strongly to glucagon, and muscle adenylate cyclase responds more strongly to adrenaline. cAMP decomposition into AMP is catalyzed by the enzyme phosphodiesterase. In the Human Metabolome Database there are 166 metabolic enzymes listed that convert cAMP⁵.



Other biological actions of cAMP include regulation of innate immune functioning⁶, axon regeneration⁷, cancer⁸, and inflammation⁹.

1. Sutherland, E. W. and Rall, T. W. Fractionation and Characterization of a Cyclic Adenine Ribonucleotide Formed by Tissue Particles. *J. Biol. Chem.*, 232:1077, 1958.
2. Marsh, J.M., The Role of Cyclic AMP in Gonadal Steroidogenesis. *Biol. Reprod.*, 14:30-53, 1976.
3. Korenman, S.G. and Krall, J.F., The Role of Cyclic AMP in the Regulation of Smooth Muscle Cell Contraction in the Uterus. *Biol. Reprod.*, 16:1-17, 1977.
4. Kelley, D.J., Bhattacharyya, A., Lahvis, G.P., Yin, J.C.P., Malter, J., and Davidson, R.J., The Cyclic AMP Phenotype of Fragile X and Autism. *Neurosci. Biobehav. Rev.*, 32(8): 1533-1543, 2008.
5. <http://www.hmdb.ca/metabolites/HMDB000058>
6. Serezani, C.H., Ballinger, M.N., Aronoff, D.M., and Peters-Golden, M., Cyclic AMP. Master Regulator of Innate Immune Cell Function. *Am. J. Resp. Cell and Mol. Biol.*, 39 (2): 127, 2008.
7. Hannila, S.S., and Filbin, M.T., The role of cyclic AMP signaling in promoting axonal regeneration after spinal cord injury. *Exp. Neurol.*, 209(2): 321–332, 2008.
8. Shankar, D.B, Cheng, J.C., and Sakamoto, K.M., Role of cyclic AMP response element binding protein in human leukemias. *Cancer*, 104(9):1819-24, 2005.
9. Galea E. and Feinstein, D.L., Regulation of the expression of the inflammatory nitric oxide synthase (NOS2) by cyclic AMP. *FASEB J.*, 13:2125-2137, 1999.

ASSAY PRINCIPLE

The DetectX® Direct High Sensitivity Cyclic AMP (cAMP) Chemiluminescent ELISA Kit is designed to quantitatively measure cAMP present in lysed cells, EDTA and heparin plasma, urine, saliva and tissue culture media samples. Please read the complete kit insert before performing this assay.

For tissue samples, saliva and urine, where the levels of cAMP are expected to be relatively high, the regular format for the assay can be used. For plasma samples and some dilute cell lysates an optional acetylation protocol should be used. This kit can measure as little as 1 femtomol cAMP per sample.

The kit is unique in that all samples and standards are diluted into an acidic Sample Diluent, which contains special additives and stabilizers, for cAMP measurement. This allows plasma, urine and saliva samples to be read in an identical manner to lysed cells. Acidified samples of cAMP are stable and endogenous phosphodiesterases are inactivated in the Sample Diluent. A cAMP standard is provided to generate a standard curve for the assay and all samples should be read off the standard curve. A white microtiter plate coated with an antibody to capture sheep IgG is provided. Prior to the addition of any samples or standards a neutralizing Plate Primer solution is added to all the used wells. Standards or diluted samples, either with or without acetylation, are pipetted into the primed wells. A cAMP-peroxidase conjugate is added to the standards and samples in the wells. The binding reaction is initiated by the addition of a sheep antibody to cAMP to each well. After a 2 hour incubation, the plate is washed and the chemiluminescent substrate is added. The substrate reacts with the bound cAMP-peroxidase conjugate to produce light. The generated light is detected in a microtiter plate reader capable of reading luminescence. The concentration of the cAMP in the sample is calculated, after making suitable correction for the dilution of the sample, using software available with most plate readers.

RELATED PRODUCTS

Kits	Catalog No.
Acetylcholinesterase Fluorescent Activity Kit	K015-F1
Butyrylcholinesterase Fluorescent Activity Kit	K016-F1
Corticosterone Chemiluminescent ELISA Kits	K014-C1/C5
Corticosterone ELISA Kits	K014-H1/H5
Cortisol ELISA Kits	K003-H1/H5
Cyclic AMP Direct ELISA Kits	K019-H1/H5
Cyclic GMP Direct ELISA Kit – Improved Sensitivity	K065-H1/H5
Prostaglandin E2 ELISA Kits	K051-H1/H5
Protein Kinase A (PKA) Colorimetric Activity Kit	K027-H1



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SUPPLIED COMPONENTS

Coated White 96 Well Plates

White plastic microtiter plate(s) coated with donkey anti-sheep IgG.

Kit K019-C1 or -C5

1 or 5 Each

Catalog Number X063-1EA

Cyclic AMP Standard

Cyclic AMP at 1,500 pmol/mL in a special stabilizing solution.

125 µL

Catalog Number C066-125UL

DetectX® Cyclic AMP Antibody

A sheep antibody specific for cyclic AMP.

Kit K019-C1 or -C5

3 mL or 13 mL

Catalog Number C064-3ML or -13ML

DetectX® Cyclic AMP Conjugate Concentrate

A 50X cyclic AMP-peroxidase conjugate concentrate stock in a special stabilizing solution.

Kit K019-C1 or -C5

60 µL or 260 µL

Catalog Number C067-60UL or -260UL

Conjugate Diluent

Contains special stabilizers and additives.

Kit K019-C1 or -C5

3 mL or 13 mL

Catalog Number X076-3ML or -13ML

Sample Diluent Concentrate

A 4X concentrate containing stabilizers and additives that must be diluted with deionized or distilled water. **CAUSTIC.**

Kit K019-C1 or -C5

12 mL or 60 mL

Catalog Number X074-12ML or -60ML

Plate Primer

A neutralizing solution containing special stabilizers and additives.

25 mL

Catalog Number X073-25ML

Acetic Anhydride

Acetic Anhydride **WARNING: Corrosive Lachrymator**

2 mL

Catalog Number X071-2ML

Triethylamine

Triethylamine **WARNING: Corrosive Lachrymator**

4 mL

Catalog Number X072-4ML

Wash Buffer Concentrate

A 20X concentrate that must be diluted with deionized or distilled water.

Kit K019-C1 or -C5

30 mL or 125 mL

Catalog Number X007-30ML or -125ML

Substrate Solution A

Kit K019-C1 or -C5

6mL or 28 mL

Catalog Number X077-6ML or -28ML

Substrate Solution B

Kit K019-C1 or -C5

6mL or 28 mL

Catalog Number X078-6ML or -28ML

Plate Sealer

Kit K019-C1 or -C5

1 or 5 Each

Catalog Number X002-1EA

STORAGE INSTRUCTIONS

All components of this kit should be stored at 4°C until the expiration date of the kit.

OTHER MATERIALS REQUIRED

Distilled or deionized water.

Repeater pipet and disposable tips capable of delivering 25 and 100 μ L.

Glass test tubes.

Microplate shaker.

96 well microplate reader capable of reading glow chemiluminescence. A list of some models of suitable readers can be found on our website at www.ArborAssays.com/resources/#general-info. All luminometers read Relative Light Units (RLU). These RLU readings will vary with make or model of plate reader.

The number of RLUs obtained is dependant on the sensitivity and gain of the reader used. If you are unsure of how to properly configure your reader contact your plate reader manufacturer or carry out the following protocol:

Dilute 5 μ L of the Cyclic AMP Conjugate Concentrate into 995 μ L of deionized water. Pipet 5 μ L of diluted conjugate into 245 μ L of deionized water. Pipet 5 μ L of this mixture into a white well and add 100 μ L of prepared CLIA substrate (see page 9 for details). This well will give you an intensity slightly above the maximum binding for the assay. Adjust the gain or sensitivity so that your reader is giving close to the readers maximum signal.

To properly analyze the data software will be required for converting raw RLU readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

PRECAUTIONS

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product. This kit utilizes a peroxidase-based readout system. Buffers, including other manufacturers Wash Buffers, containing sodium azide will inhibit color production from the enzyme. Make sure **all** buffers used for samples are **azide free**. Ensure that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer as prepared on page 9.

The antibody coated plate needs to be stored desiccated. The silica gel pack included in the foil ziploc bag will keep the plate dry. The silica gel pack will turn from blue to pink if the ziploc has not been closed properly.

The supplied Sample Diluent Concentrate is acidic. Take appropriate precautions when handling the reagent.

The kit uses acetic anhydride and triethylamine as acetylation reagents. Triethylamine and acetic anhydride are lachrymators. **Caution: corrosive, flammable, and harmful vapor. Use in hood with proper ventilation and wear appropriate protective safety wear.**



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SAMPLE TYPES

This assay has been validated for lysed cells, saliva, urine, EDTA and heparin plasma tissue samples and for tissue culture media samples. Samples should be stored at -70°C for long term storage. 24-Hour urine samples may need to have 1 mL concentrated hydrochloric acid added for every 100 mL volume to act as a preservative. Samples containing visible particulate should be centrifuged prior to using.

Cyclic AMP is identical across all species and we expect this kit may measure cAMP from sources other than human. The end user should evaluate recoveries of cAMP in other samples being tested.

After dilution in the Sample Diluent (see page 9) there may be some precipitation of proteins. This precipitate will not effect the results obtained. After being diluted in Sample Diluent the samples can be assayed directly within 2 hours, or frozen at $\leq -70^{\circ}\text{C}$ for later analysis. Severely hemolyzed samples should not be used in this kit.

For samples containing low levels of cAMP and for all plasma samples, the acetylated assay protocol must be used due to its enhanced sensitivity. **All standards and samples should be diluted in glass test tubes.**

SAMPLE PREPARATION

Cells

Cell lysis buffers containing high concentrations of SDS or other detergents may not be compatible with this assay or may require extra dilution. Please read Interferents section on page 22 for more information.

This kit is compatible with either adherent or non-adherent cells. The cells can be grown in any suitable sterile containers such as Petri dishes, 12-, 48- or 96-well culture plates or flasks. The cells must be isolated from the media prior to being lysed with the provided Sample Diluent. The acidic Sample Diluent contains detergents to lyse the cells, inactivate endogenous phosphodiesterases and stabilize the cAMP. Some cell types are extremely hardy and the end user should optimize the lysis conditions utilizing freeze-thaw cycles and ultrasonic treatments to fully lyse their cells.

We used $\sim 10^7$ Jurkat cells per mL of Sample Diluent. Cell number needs to be determined by the end user since it will be dependant on cell type and treatment conditions. Care must be taken not to over dilute the samples.

For adherent cells, the media should be aspirated from the cells and the cells washed with PBS. The adherent cells should be treated directly with the Sample Diluent for 10 minutes at room temperature. Cells can be scraped to dislodge them from the plate surface and cells should be inspected to ensure lysis. Detergent has been added to the Sample Diluent to help lysis occur. Centrifuge the samples at $\geq 600 \times g$ at 4°C for 15 minutes and assay the supernatant directly. If required, the TCM can be assayed for cAMP as outlined below.

For non-adherent cells, pellet and wash the cells with PBS by centrifuging the samples at $\geq 600 \times g$ at 4°C for 15 minutes as described above. Treat the aspirated, washed pellet directly with the Sample Diluent for 10 minutes at room temperature. Cells should be inspected to ensure lysis. Detergent has been added to the Sample Diluent to help lysis occur. Centrifuge the samples at $\geq 600 \times g$ at 4°C for 15 minutes and assay the supernatant directly. If required, the TCM can be assayed for cAMP as outlined below.

Tissue Samples

Tissues samples should be frozen in liquid nitrogen and stored at -80°C if analysis is not to be carried out immediately.

Grind the frozen tissue in a stainless steel mortar under liquid nitrogen until it is a fine powder. Allow the liquid nitrogen to evaporate and weigh the powdered tissue. Add 1 mL of Sample Diluent for every 100 mg of tissue. Incubate in the Sample Diluent for 10 minutes on ice, and then centrifuge at $\geq 600 \times g$ at 4°C for 15 minutes. Collect the supernatant and run in the assay immediately or store frozen at $\leq -70^{\circ}\text{C}$.

For samples that require concentration and delipidation, a trichloroacetic acid (TCA)/ether protocol can be used. Grind the frozen tissue in a stainless steel mortar under liquid nitrogen until it is a fine powder. Allow the liquid nitrogen to evaporate and weigh the powdered tissue. Add 1 mL of ice cold 5% TCA (weight/volume) for every 100 mg of tissue and grind in a glass-Teflon mortar. Incubate in the TCA for 10 minutes on ice, and then centrifuge at $\geq 600 \times g$ at 4°C for 15 minutes. Collect the supernatant.

For every 1 mL of TCA supernatant add 3 mL of water saturated diethyl ether* and shake in a glass vial. Allow the ether to separate as the top layer, remove it and discard the ether. Dry the aqueous layer by lyophilization or using a vacuum centrifuge. Reconstitute by adding 1 mL of Sample Diluent for every mL of 5% TCA used to extract and run in the assay immediately or store at $\leq -70^{\circ}\text{C}$.

***Diethyl ether is extremely flammable and should be used in a hood.**

Tissue Culture Media

For measuring cAMP in tissue culture media (TCM), samples should be read off a standard curve generated in TCM. Samples may need to be diluted further in TCM. We have validated the assay using RPMI-1640.

Plasma Samples

Plasma samples should be diluted $\geq 1:10$ with the supplied Sample Diluent and acetylated prior to running in the Acetylated Format assay (pages 14-16).

Urine Samples

Urine samples should be diluted $\geq 1:20$ with the supplied Sample Diluent prior running in the assay. Due to the high concentration of cAMP in urine, samples may need to be diluted further.

Saliva Samples

Saliva samples should be diluted $\geq 1:4$ with the supplied Sample Diluent prior running in the assay. See our Saliva Sample Handling Instructions at www.ArborAssays.com/assets/saliva-sample-protocol.pdf

Use all samples within 2 hours of dilution in Sample Diluent.



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REAGENT PREPARATION

Allow the kit reagents to come to room temperature for 30-60 minutes. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Wash Buffer

Dilute Wash Buffer Concentrate 1:20 by adding one part of the concentrate to nineteen parts of deionized water. Once diluted this is stable at room temperature for 3 months.

Sample Diluent

Prepare the Sample Diluent by diluting the Sample Diluent Concentrate 1:4, adding one part of the concentrate to three parts of deionized water. Once diluted this is stable at 4°C for 3 months.

Cyclic AMP Conjugate

The supplied Cyclic AMP Conjugate Concentrate should be diluted 1:50 with the Conjugate Diluent as indicated in the table below. Once diluted the Cyclic AMP conjugate is stable for one month when stored at 4°C.

	1 Plate	2 Plates	3 Plates	4 Plates	5 Plates
Conjugate Concentrate	50 µL	100 µL	150 µL	200 µL	250 µL
Conjugate Diluent	2.45 mL	4.9 mL	7.35 mL	9.8 mL	12.25 mL
Final Mixture	2.5 mL	5 mL	7.5 mL	10 mL	12.5 mL

Chemiluminescent Substrate

Mix one part of the Substrate Solution A with one part of Substrate Solution B in a brown bottle. Once mixed the substrate is stable for one month when stored at 4°C.

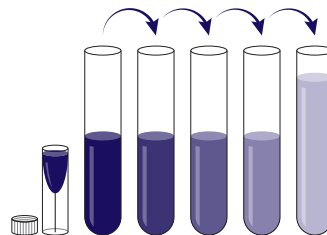
REAGENT PREPARATION - REGULAR FORMAT

Use this format for urine, saliva and some cell lysates. Do **not** use for plasma samples.

All standards and samples should be diluted in **glass** test tubes.

Standard Preparation - Regular Format

Label one glass test tube as Stock 2 and five tubes as #1 through #5. Pipet 90 μL of Sample Diluent into the Stock 2 tube and 450 μL of Sample Diluent into tube #1. Pipet 300 μL of Sample Diluent into tubes #2 to #5. The Cyclic AMP stock solution contains an organic solvent. Prerinse the pipet tip several times to ensure accurate delivery. Carefully add 10 μL of the cAMP stock solution to the Stock 2 tube and vortex completely. Take 50 μL of the cAMP solution in the Stock 2 tube and add it to tube #1 and vortex completely. Repeat the serial dilutions for tubes #3 through #5. The concentration of Cyclic AMP in tubes 1 through 5 will be 15, 5, 1.667, 0.556, and 0.185 pmol/mL.



Non-Acetylated	Stock 2	Std 1	Std 2	Std 3	Std 4	Std 5
Sample Diluent (μL)	90	450	300	300	300	300
Addition	Cyclic AMP Std.	Stock 2	Std 1	Std 2	Std 3	Std 4
Vol of Addition (μL)	10	50	150	150	150	150
Final Conc (pmol/mL)	150	15	5	1.667	0.556	0.185

Use Standards within 1 hour of preparation.



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ASSAY PROTOCOL - REGULAR FORMAT

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine cAMP concentrations.

1. Use the plate layout sheet on the back page to aid in proper sample and standard identification. Determine the number of wells to be used and return unused wells to the foil pouch with desiccant. Seal the ziploc plate bag and store at 4°C.
2. Add 50 µL of Plate Primer into all wells used. **Failure to add plate primer to all wells first will cause assay to fail.**
3. Pipet 75 µL Sample Diluent into the non-specific binding (NSB) wells.
4. Pipet 50 µL of Sample Diluent into the maximum binding (B0 or Zero standard) wells.
5. Pipet 50 µL of samples or standards into wells in the plate.
NOTE: Sample Diluent will turn from orange to bright pink upon sample or standard addition to the Plate Primer in the wells.
6. Add 25 µL of the diluted DetectX® cAMP Conjugate to each well using a repeater pipet.
7. Add 25 µL of the DetectX® cAMP Antibody to each well, **except the NSB wells**, using a repeater pipet.
8. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and shake at room temperature for 2 hours. We recommend shaking at around 700–900 rpm. If the plate is not shaken, signals bound will be approximately 25% lower.
9. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.
10. Add 100 µL of the mixed Chemiluminescent Substrate to each well, using a repeater pipet.
11. Incubate the plate at room temperature for 5 minutes without shaking.
12. Read the luminescence generated from each well in a mutimode or chemiluminescent plate reader using a 0.1 second read time per well. The chemiluminescent signal will decrease about 40% over 60 minutes.
13. Use the plate reader's built-in 4PLC software capabilities to calculate cAMP concentration for each sample.

NOTE: *If you are using only part of a strip well plate, at the end of the assay throw away the used wells and retain the plate frame for use with the remaining unused wells.*

CALCULATION OF RESULTS

All luminometers read Relative Light Units (RLU). These RLU readings will vary with make or model of plate reader. Average the duplicate RLU readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean RLU's for the NSB. The sample concentrations obtained, calculated from the %B/B0 curve, should be multiplied by the dilution factor to obtain neat sample values.

Or use the online tool from MyAssays to calculate the data:

www.myassays.com/arbor-assays-cyclic-amp-direct-chemiluminescent-eia-kit-non-acetyl.assay

TYPICAL DATA - REGULAR FORMAT

Sample	Mean RLU	Net RLU	% B/B0	Cyclic AMP Conc. (pmol/mL)
NSB	22,115	0	-	-
Standard 1	49,900	27,785	15.0	15
Standard 2	78,890	56,775	30.6	5
Standard 3	120,400	98,285	52.9	1.667
Standard 4	162,345	140,230	75.5	0.556
Standard 5	187,510	165,395	89.1	0.185
B0	207,840	185,725	100	0
Sample 1	67,910	45,795	24.7	7.03
Sample 2	111,565	89,450	48.2	2.09

Always run your own standard curve for calculation of results. Do not use this data.



*The MyAssays logo is a registered trademark of MyAssays Ltd.

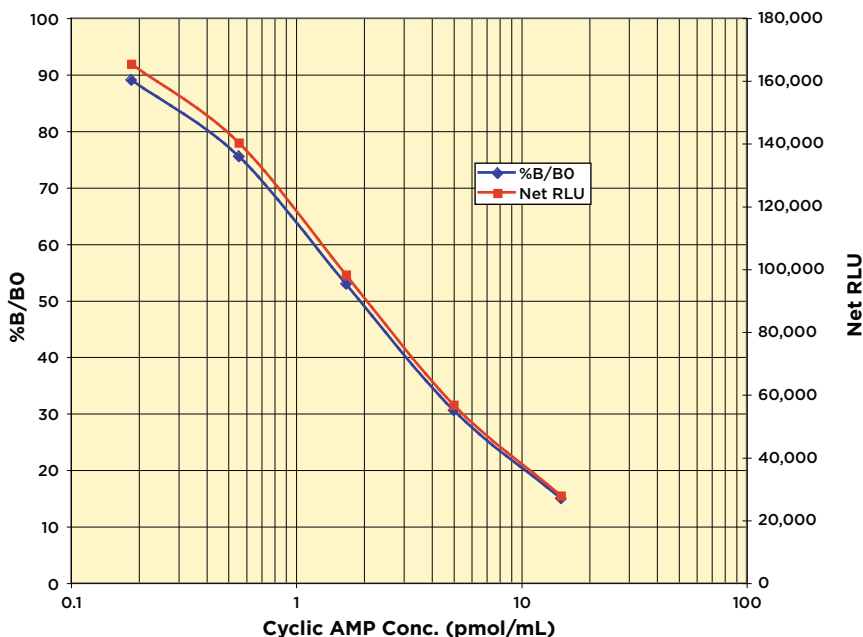
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EXPECT ASSAY ARTISTRY™

Typical Standard Curve - Regular Format



Always run your own standard curve for calculation of results. Do not use this data.

VALIDATION DATA - REGULAR FORMAT

Sensitivity and Limit of Detection

Sensitivity was calculated by comparing the RLU's for eighteen wells run for each of the B0 and standard #5. The detection limit was determined at two (2) standard deviations from the B0 along the standard curve. **Sensitivity was determined as 0.119 pmol/mL. This is equivalent to 5.95 fmol cAMP per well.**

The Limit of Detection for the assay was determined in a similar manner by comparing the RLU's for twenty runs for each of the zero standard and a low concentration human urine sample. **Limit of Detection was determined as 0.076 pmol/mL. This is equivalent to 3.8 fmol cAMP per well.**

ACETYLATED PROTOCOL - OVERVIEW

This format must be used for plasma, some cell lysates and any sample with low cAMP concentrations.

Prior to running the acetylated assay, all standards, samples and the Sample Diluent used for the B0 and NSB wells must be acetylated. Acetylation is carried out by adding 15 μ L of the Acetylation Reagent (as prepared below) for each 300 μ L of the standard, sample and Sample Diluent. After addition of the Acetylation Reagent immediately vortex each treated standard, sample or Sample Diluent and use within 30 minutes of preparation.

Note: Upon Acetylation, all of the standards and samples diluted in the **orange** Sample Diluent will change to a pale **yellow** color.

REAGENT PREPARATION - ACETYLATED FORMAT

Acetylation Reagent

Working in a fume hood mix one part of Acetic Anhydride with 2 parts of Triethylamine in a glass test tube. Use the following table to help determine the amount of Acetylation Reagent to make.

Reagents	Number of Samples to be Tested			
	20	40	100	200
Acetic Anhydride Volume (μ L)	200	400	1,000	2,000
Triethylamine Volume (μ L)	400	800	2,000	4,000
Acetylation Reagent Vol (mL)	0.6	1.2	3	6

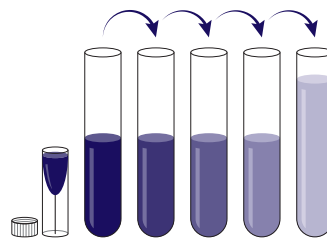
Use the Acetylation Reagent within 60 minutes of preparation.



Standard Preparation - Acetylated

All standards and samples should be diluted in glass test tubes.

Label one tube as Stock 2. Label eight glass test tubes as #1 through #8. Pipet 290 μL of Sample Diluent into the Stock 2 tube. Pipet 900 μL of Sample Diluent into tube #1 and 400 μL into tubes #2 to #8. **The Cyclic AMP stock solution contains an organic solvent. Prerinse the pipet tip several times to ensure accurate delivery.** Carefully add 10 μL of the cAMP stock solution to the Stock 2 tube and vortex completely. Carefully add 100 μL of the Stock 2 tube to tube #1 and vortex completely. Take 400 μL of the cAMP solution in tube #1 and add it to tube #2 and vortex completely. Repeat the serial dilutions for tubes #3 through #8. The concentration of Cyclic AMP in tubes 1 through 8 will be 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, and 0.039 pmol/mL.



	Stock 2	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8
Sample Diluent (μL)	290	900	400	400	400	400	400	400	400
Addition	cAMP Std.	Stock 2	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
Vol of Addition (μL)	10	100	400	400	400	400	400	400	400
Final Conc (pmol/mL)	50	5	2.5	1.25	0.625	0.313	0.156	0.078	0.039

Standard and Sample Acetylation

Pipet 300 μL of Sample Diluent into a glass tube to act as the Zero standard/NSB tube. Add 15 μL of Acetylation Reagent to this tube and vortex immediately. Proceed to assay within 30 minutes.

Pipet 300 μL of each standard and sample to be tested into glass tubes. Add 15 μL of the Acetylation Reagent into each tube and vortex immediately prior to proceeding to next tube. Proceed to assay within 30 minutes.

Note: Samples and Sample Diluent will turn from **orange** to pale **yellow** upon acetylation.

Use Acetylated Standards and Samples within 30 minutes of preparation.

ASSAY PROTOCOL - ACETYLATED FORMAT

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine cAMP concentrations.

1. Use the plate layout sheet on the back page to aid in proper sample and standard identification. Determine the number of wells to be used and return unused wells to the foil pouch with desiccant. Seal the ziploc plate bag and store at 4°C.
2. Add 50 µL of Plate Primer into all wells used. **Failure to add plate primer to all wells first will cause assay to fail.**
3. Pipet 75 µL acetylated Sample Diluent into the non-specific binding (NSB) wells.
4. Pipet 50 µL of acetylated Sample Diluent into the maximum binding (B0 or Zero standard) wells.
5. Pipet 50 µL of acetylated samples or standards into wells in the plate.
6. Add 25 µL of the diluted DetectX® cAMP Conjugate to each well using a repeater pipet.
7. Add 25 µL of the DetectX® cAMP Antibody to each well, **except the NSB wells**, using a repeater pipet.
8. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and shake at room temperature for 2 hours. We recommend shaking at around 700–900 rpm. If the plate is not shaken, signals bound will be approximately 25% lower.
9. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.
10. Add 100 µL of the mixed Chemiluminescent Substrate to each well, using a repeater pipet.
11. Incubate the plate at room temperature for 5 minutes without shaking.
12. Read the luminescence generated from each well in a mutimode or chemiluminescent plate reader using a 0.1 second read time per well. The chemiluminescent signal will decrease about 40% over 60 minutes.
13. Use the plate reader's built-in 4PLC software capabilities to calculate cAMP concentration for each sample.

NOTE: *If you are using only part of a strip well plate, at the end of the assay throw away the used wells and retain the plate frame for use with the remaining unused wells.*

CALCULATION OF RESULTS

All luminometers read Relative Light Units (RLU). These RLU readings will vary with make or model of plate reader. Average the duplicate RLU readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean RLU's for the NSB. The sample concentrations obtained, calculated from the %B/B0 curve, should be multiplied by the dilution factor to obtain neat sample values.

Or use the online tool from

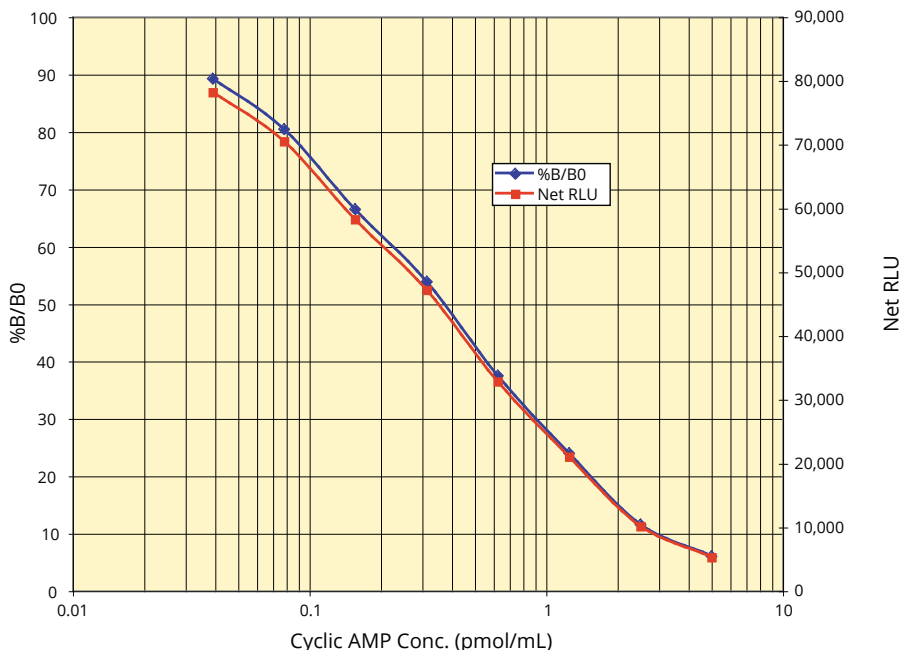
www.myassays.com/arbor-assays-cyclic-amp-direct-chemiluminescent-eia-kit-non-acetyl.assay

TYPICAL DATA - ACETYLATED

Sample	Mean RLU	Net RLU	% B/B0	Cyclic AMP Conc. (pmol/mL)
NSB	21,440	0	-	-
Standard 1	26,755	5,315	6.1	5
Standard 2	31,575	10,135	11.6	2.5
Standard 3	42,490	21,050	24.0	1.25
Standard 4	54,320	32,880	37.5	0.625
Standard 5	68,655	47,215	53.9	0.3125
Standard 6	79,730	58,290	66.5	0.156
Standard 7	91,955	70,515	80.5	0.078
Standard 8	99,660	78,220	89.3	0.039
B0	109,065	87,625	100.0	0
Sample 1	38,635	17,195	19.6	0.98
Sample 2	56,885	35,445	40.5	0.310

Always run your own standard curve for calculation of results. Do not use this data.

Typical Standard Curve - Acetylated



Always run your own standard curve for calculation of results. Do not use this data.

VALIDATION DATA - ACETYLATED FORMAT

Sensitivity and Limit of Detection

Sensitivity was calculated by comparing the RLU's for eighteen wells run for each of the acetylated B0 and standard #8. The detection limit was determined at two (2) standard deviations from the B0 along the standard curve. **Sensitivity was determined as 0.015 pmol/mL. This is equivalent to 0.75 fmol cAMP per sample.**

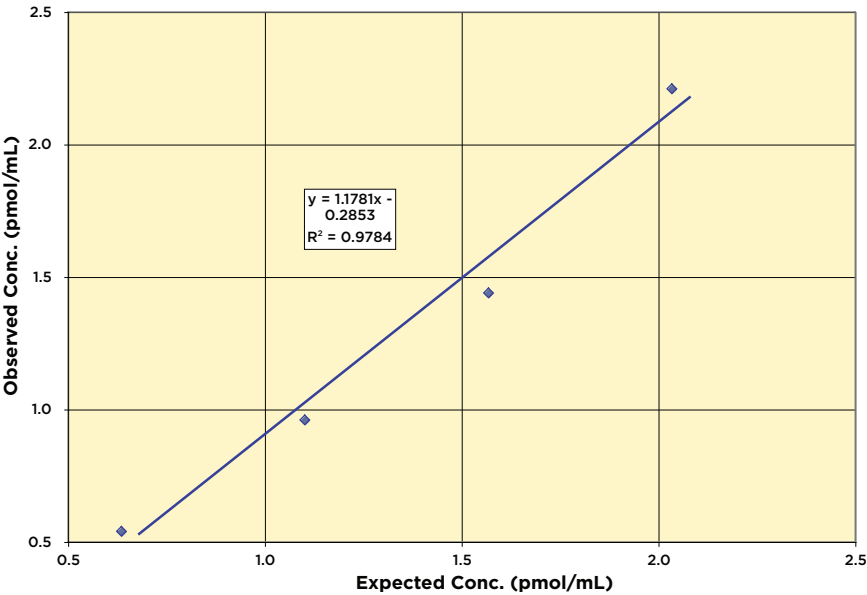
The Limit of Detection for the assay was determined in a similar manner by comparing the RLU's for twenty runs for each of acetylated zero standard and a low concentration acetylated human sample. **Limit of Detection was determined as 0.019 pmol/mL. This is equivalent to 0.95 fmol cAMP per sample.**

VALIDATION DATA - REGULAR AND ACETYLATED

Linearity - Non Acetylated

Linearity was determined by taking two Jurkat cell lysate samples, one with a low cAMP level of 0.2 pmol/mL and one with a higher level of 2.5 pmol/mL, and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

High Cell Conc.	Low Cell Conc,	Expected Conc. (pmol/mL)	Observed Conc. (pmol/mL)	% Recovery
80%	20%	2.0	2.2	108.7
60%	40%	1.6	1.4	91.8
40%	60%	1.1	1.0	87.1
20%	80%	0.6	0.5	84.9
Mean Recovery				93.1%



Intra Assay Precision - Regular

Two human urine samples were diluted with Sample Diluent and run in replicates of 20 in an assay. The mean and precision of the calculated cAMP concentrations were:

Sample	Cyclic AMP Conc. (pmol/mL)	%CV
1	11.2	3.9
2	3.6	8.3

Inter Assay Precision - Regular

Two human urine samples were diluted with Sample Diluent and run in duplicates in fourteen assays run over multiple days by four operators. The mean and precision of the calculated cAMP concentrations were:

Sample	Cyclic AMP Conc. (pmol/mL)	%CV
1	6.5	8.1
2	2.1	13.0

Intra Assay Precision - Acetylated

Two human plasma samples were diluted with Sample Diluent, acetylated and run in replicates of 20 in an assay. The mean and precision of the calculated cAMP concentrations were:

Sample	Cyclic AMP Conc. (pmol/mL)	%CV
1	1.32	8.4
2	0.39	10.9

Inter Assay Precision - Acetylated

Two human plasma sample were diluted with Sample Diluent, acetylated and run in duplicates in fourteen assays run over multiple days by four operators. The mean and precision of the calculated cAMP concentrations were:

Sample	Cyclic AMP Conc. (pmol/mL)	%CV
1	1.02	10.1
2	0.32	16.5



SAMPLE VALUES

Fourteen human plasma samples were tested in the assay. Diluted samples were acetylated and run in the Acetylated Format. Values ranged from 12.5 to 43.32 pmol/mL with an average for the samples of 21.15 pmol/mL. The normal reference range for cAMP in plasma is 3.9-13.7 pmol/mL¹⁰. Four human urine samples were diluted > 1:30 in Sample Diluent and values ranged in the neat samples from 1,099 to 4,585 pmol/mL with an average for the samples of 3,034 pmol/mL. The normal reference range for cAMP in urine is 800-12,000 pmol/mL¹¹. Two human saliva samples were diluted 1:4 in Sample Diluent and run in both the Regular and Acetylated Formats. Values ranged from 5.8 to 6.6 pmol/mL with an average of 6.2 pmol/mL in the neat samples. The normal range for cAMP in saliva is 3.4-17.2 pmol/mL¹².

10. NIH Clinical Center,
<http://cclnprod.cc.nih.gov/dlm/testguide.nsf/Index/EB6E90F8D951346F85256BA4004C96E4?OpenDocument>
11. NIH Clinical Center,
<http://cclnprod.cc.nih.gov/dlm/testguide.nsf/Index/24B381AEE513EB8785256BA40052ADAD?OpenDocument>
12. Sproles, A.C., Cyclic AMP Concentration in Saliva of Normal Children and Children with Down's Syndrome, J. Dent. Res., 1976, 52, 915-917.

CROSS REACTIVITY

The following cross reactants were tested in the assay and calculated at the 50% binding point.

Nucleotide	Cross Reactivity (%)
Cyclic AMP	100%
AMP	< 0.08%
GMP	< 0.08%
Cyclic GMP	< 0.08%
ATP	< 0.08%

INTERFERENTS

A variety of detergents were tested as possible interfering substances in the assay. CHAPS and Tween 20 at 0.1% increased measured cAMP by 8.9% and decreased measured cAMP by 0.9%, respectively. Triton X-100 at 2% increased measured cAMP by 1.8% and CTAC at 0.05% increased measured cAMP by 6.3%. Samples containing SDS above 0.01% should not be used in the assay.

LIMITED WARRANTY

Arbor Assays warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

We must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if we are not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

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